Genetic Aspects of Hepatocellular Carcinogenesis

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ABSTRACT: *Hepatocellular carcinoma (HCC) is linked etiologically to viruses (hepatitis B virus [HBV] and hepatitis C virus [HCV]), chemical carcinogens (i.e., aflatoxins), and other environmental and host factors causing chronic liver injury. Some hepatoblastomas may be linked to inherited gene mutations, but adult hereditary HCC appears to be rare. HCCs display gross genomic alterations, including DNA rearrangements associated with HBV DNA integration, loss of heterozygosity, and, less importantly, chromosomal amplifications and loss of imprinting. Many genes with somatic mutations have now been identified in these tumors. Most frequently involved genes are tumor suppressor genes such as* p53, M6P/IGF2R, *β-catenin,* p16INK4A, *and retinoblastoma genes. Most identified mutations are somatic, but germline mutations of* p16INK4A, APC, *and* BRCA2 *have also been reported. Oncogenic activation of several cellular genes such as cyclin D and cyclin A have been described in HCC, but the possible implication of candidate viral oncogenes (i.e., X protein of HBV) is still debated. A comprehensive analysis of all the genetic changes described for HCC demonstrates that at least four different growth regulatory pathways are altered in these tumors. However, each pathway appears to be implicated in a limited fraction of these tumors, suggesting that HCCs are genetically heterogenous neoplasms. This genetic heterogeneity correlates with the heterogeneity of etiologic factors implicated in HCC.*

KEY WORDS: hepatocellular carcinoma, primary liver cancer, *p53, p16INK4A,* cyclin D, β-catenin, *M6P/IGF2R*

Hepatocellular carcinoma (HCC) cells often display chromosomal changes such as polyploidy, loss of heterozygosity (LOH), allelic imbalance (AI), amplifications, and translocations.[1] It has also been known for a long time that hepatitis B virus (HBV) DNA causes chromosomal rearrangements by integration into the host genome.[2] It is expected that the chromosomal regions that undergo tumor-specific changes harbor critical genes involved directly (oncogenes and tumors suppressor genes) or indirectly (DNA repair genes) in carcinogenesis. To date, a dozen genes, including *p53,* mannose-6-phosphate/insulin-like factor 2 receptor (*M6P/ IGF2R*), β-catenin, retinoblastoma (*RB1*), *p16INK4A,* adenomatosis polyposis coli (*APC*), breast cancer gene 2 (*BRCA2*), cyclin A, cyclin D, and insulin-like growth factor 2 (IGF2) have been shown to be altered in HCC and/or hepatoblastoma (Table 1). This list will probably grow over the next years to include many more genes. There are at least two reasons to explain the high number of altered genes in HCC. First, solid tumors of the adult may need the accumulation of many genetic alterations before they become clinically detectable. Indeed,

Objectives
Upon completion of this article, the reader should be able to: 1) list the factors that are etiologically linked to hepatocellular carcinoma; 2) state the most frequently involved genes; and 3) recognize the four different growth regulatory pathways that are altered in these tumors.

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TABLE 1. Genetic Alterations in Hepatocellular Carcinoma and Hepatoblastoma

Gene	*Mutation (%)*	*Other Alterations*	*References*
p53	28	HBx interaction	see Table 2
M6P/IGF2R	18–33	HD	21, 36, 44
TGFRB2	0	—	23
RB1	15	LOH, HD	11,‡ 45–50
p15INK4B	0	HD	50, 51
*p16INK4A**	0–55	HD, Methylation	50–54
p21	5	—	55
Cyclin D†	11–13	—	56, 57
Cyclin A†	19	HBV integration	58, 59
β–catenin	19–26	—	30, 31
*APC**	62‡	LOH	11, 32–35, 37
E-cadherin	NR	LOH, Methylation	60, 61
*BRCA2**	5	LOH	62
Smad2	0–2	—	24, 25
Smad4	0–6	—	24, 25
hMLH1	NR	LOH	63
hMSH2	NR	LOH	63
IGF2	NR	LOI	38–43
K-*ras*	0–17	—	90–95
N-*ras*	0–16	Amplification	90, 91, 93, 95, 96
H-*ras*	0–10	Methylation	73, 91, 93, 95, 97, 98
c-*myc*†	0–50	—	99, 100
N-*myc*†	0	—	99, 101

*Somatic and germline mutations.
†Amplification.
‡In hepatoblastoma only.
HD, homozygous deletion.
LOH, loss of heterozygosity.
LOI, loss of imprinting.

the well-known "latent period" between the first exposure to an etiologic agent (i.e., infection with HBV) and the development of HCC is in favor of such a hypothesis.[3] Second, the multiplicity of genetic alterations in HCC may indicate that different etiologic factors affect different sets of target genes in hepatocytes. This etiologically defined genetic heterogeneity of HCC results in a phenotypic heterogeneity of these tumors. In other words, distinct but related growth regulatory pathways are altered during hepatocarcinogenesis. As discussed later, at least four different pathways are altered in human HCCs.

p53 GENE

Many reports now indicate that the *p53* gene, which is located on chromosome 17p, is mutated in about 30% of HCCs worldwide (for a recent review, see ref. 4). All reported *p53* mutations in HCC are somatic. Therefore, germline mutations of *p53* appear not to predispose to HCC. Both the frequency and the type of *p53* mutations are different depending on geographic location and suspected etiology of these tumors (Table 2). An HCC-specific codon 249 mutation (AGG → AGT) leading to an arginine to serine substitution (R249S), suspected to be induced by aflatoxins, was found in most HCCs from geographic areas with high incidence of HCC and a high risk of exposure to aflatoxins.[5–7] This mutation was found in 50% of HCCs from Mozambique,[8,9] 50 to 75% of HCCs from Qidong province of China,[6,10,11] and 67% of HCCs from Senegal.[7] A worldwide study by Ozturk et al.[8] suggested a close correlation between the presence of codon 249 mutations in HCC and high risk of aflatoxin intake. This early study has now been largely confirmed by others. As shown in Table 1, the codon 249 mutation is present in 36% of tumors from Africa and 32% of tumors from China, respectively. These two regions of the world are known for high incidence of HCC, where both HBV and aflatoxins are recognized as major etiologic factors. In contrast, the codon 249 mutation is seen in less than 4% of HCCs from Japan, Europe, and North America, where HBV and hepatitis C virus (HCV), but not aflatoxins, are the main etiologic factors. The overall frequency of codon 249 mutations in the world is 11%. Mutations affecting other codons of the *p53* gene are detected in HCC, and their worldwide frequency is 18%. The frequency of all *p53* mutations in HCC varies between 15% in Europe and 42% in China, with a worldwide frequency of 27% (see Table 2 for a detailed analysis of *p53* mutations). Thus, *p53* gene is mutated in about a third of HCCs, but only a third of these mutations can be etiologically linked to a high risk of aflatoxin exposure. Therefore, *p53* mutations can occur in HCC independent of aflatoxin risk, and in HBV or HCV infection. However, Unsal et al.[9] reported an

TABLE 2. Frequency of *p53* Mutations in Hepatocellular Carcinoma

Region	p53–*249ser*	p53-*other*	p53-*total*
North America	4/89*	6/34	6/34
	(4%)	(18%)	(18%)
South America†	3/16	ND	ND
	(19%)	—	—
Europe	0/131	13/109	23/151
	(0%)	(12%)	(15%)
Africa‡	19/53	2/26	11/26
	(36%)	(8%)	(42%)
China§	62/191	16/132	50/132
	(32%)	(12%)	(38%)
Japan	7/292	53/232	94/353
	(2%)	(23%)	(27%)
Other‖	20/265	57/265	79/273
	(8%)	(22%)	(29%)
Total	115/1037	147/798	263/969
	(11%)	(18%)	(27%)

Data were compiled from references 6, 7, 9–11, 45, 64–89, 98 and 102–104. The numbers do not add up for two reasons: in some studies only the *p53*-249 mutation was reported; in some others only the information of the total number of mutations was reported.
*U.S.A. including Alaska.
†Mexico only.
‡South Africa, Mozambique, and Senegal only.
§Mainland China and Hong Kong.
‖Australia, Singapore, South Korea, Taiwan, Thailand.

apparent association between the presence of X gene coding sequences of HBV (HBx) and wild-type *p53* in HCC. Based on this observation, a possible interference of HBV with wild-type *p53* function was suggested.[9] Indeed, recent studies showed that HBx protein encoded by the X region of HBV interacts with wild-type *p53* protein both physically and functionally.[12–15] These observations suggest that the suspected oncogenic activity of HBx protein is linked to functional inactivation of wild-type *p53* protein, as observed with other viral proteins with transforming activity. However, the interaction of HBx with *p53* was shown only experimentally. It is presently unclear whether HBx–*p53* interactions really occur in HBV-infected hepatocytes and/or in HCC cells with integrated HBV DNA sequences.

Frequent involvement of *p53* mutations in HCC is not surprising for several reasons. First, the *p53* gene is the only known gene to be mutated at a very high frequency in tumors of different origin.[16] Second, this protein is involved in different cellular processes (cell cycle arrest, apoptosis, differentiation, angiogenesis, etc.), all critically involved in the development of malignancy.[17] Under physiologic conditions, p53 protein is complexed with MDM2 protein that promotes a rapid degradation of *p53*. MDM2–*p53* complexes are inhibited either by p19ARF (induced by both cellular and viral oncogenes) or by N-terminal phosphorylation of *p53* by DNA-dependent protein kinase. This leads to an accumulation and functional activation of *p53* in cells, leading to either cell cycle arrest by p21 or apoptosis by *bax* induction. Thus, p53 protein appears to be involved in a growth control response to abnormal oncogene expression and DNA damage.[17] In patients with chronic liver disease, the risks of oncogene activation and DNA damage are elevated. As stated earlier, HBx may have an oncogenic activity and aflatoxins are potent DNA damaging agents.[1,2]

p16INK4A, CYCLIN D, AND RETINOBLASTOMA GENES

These three genes encode for proteins involved in the regulation of the G1 phase of the cell cycle. Cyclin D forms active complexes with CDK4 protein, whereas p16 protein is an inhibitor of CDK4 activity.[18] The retinoblastoma protein (pRb) is the main known substrate of CDK4. In nonproliferating cells, pRb protein forms complexes with E2F transcription factors. When complexed to pRb, E2Fs are transcriptionally inactive. Upon phosphorylation by CDK4, pRb is released from its complexes and "free E2Fs" promote the initiation of DNA synthesis.[19] These observations predict that the loss of pRb protein or its aberrant phosphorylation will lead to a loss of growth control at the G1 phase of the cell cycle. Increased phosphorylation of pRb may result from an aberrant activation of CDK4 by either an excess of cyclin D and/or a deficit in p16 protein. Recent studies demonstrated that all three genes, namely *RB1*,

p16INK4A, and cyclin D, undergo structural alterations in HCC. The retinoblastoma gene (*RB1*) is one of the tumor suppressor genes studied in HCC just after the implication of *p53* in these tumors. LOH at the *RB1* gene locus is quite frequent in HCC. In addition, *RB1* mutations were observed in 15% of these tumors (Table 1).

The *p16INK4A* gene, which is located at chromosome 9p, codes for two alternatively spliced transcripts.[17,18] One of the transcripts is for p16 protein, an inhibitor of cyclin-dependent kinases 4 and 6.[18] *p16INK4A* status in HCC has been studied independently by several laboratories. Both germline and somatic mutations of *p16INKA* were found in HCC patients. It was also reported that about 50% of HCC display *de novo* methylation of *p16INK4A*, as observed in other cancers (see Table 1 and references therein). It is known that *de novo* methylation is a mechanism involved in gene silencing.[20] Therefore, one can assume that HCC cells with methylated *p16INK4A* are unable to express the gene, leading to the loss of a cyclin-dependent kinase inhibitor protein.

As shown in Table 1, cyclin D and cyclin A genes were shown to be amplified in 10–20% of HCCs. It is noteworthy that *RB1*, *p16INKA*, and cyclin genes are mutated *individually* in 10 to 20% of HCCs. Although this frequency is not high, their involvement in the same growth regulatory pathway implies that when combined, these mutations will lead a loss of growth control in more than 30% of HCCs.

M6P/IGF2 RECEPTOR, *SMAD2*, AND *SMAD4* GENES

The mannose-6-phosphate/insulin-like growth factor 2 receptor (M6P/IGF2R) is involved in the activation of transforming growth factor beta (TGF-β), whereas *SMAD2* and *SMAD4* genes are intracellular mediators of TGF-β, which induces both growth inhibition and apoptotic cell death in hepatocytes.[21–23] After the demonstration of LOH at the M6P/IGF2R gene locus by De Souza et al.,[21] several reports described that the *MGP/IGF2R* gene is mutated in 18 to 33% of HCCs (Table 1). *SMAD2* and *SMAD4* genes appear to be mutated in less that 10% of these cancers.[24,25] In contrast, no mutation of TGF-β receptor type II was found in HCC.[24] Taken together, these observations demonstrate that at least three genes involved in TGF-β-mediated growth control are altered in HCC and that overall the TGF-β pathway is altered in about 25% of HCCs.

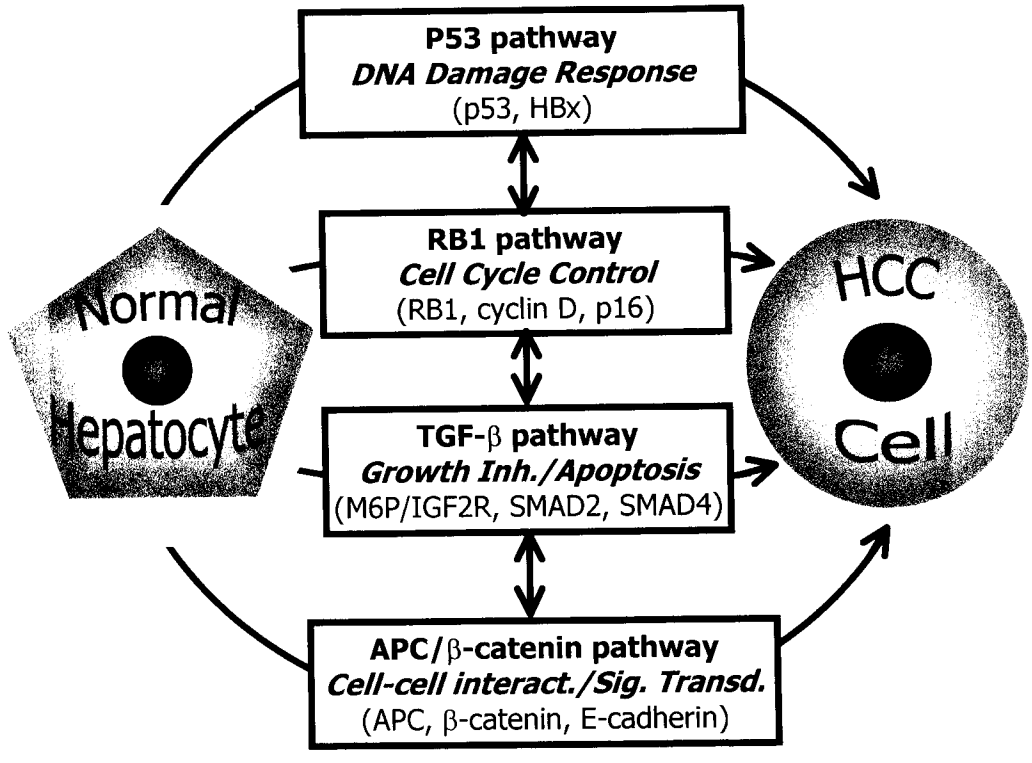

FIG. 1. Main regulatory pathways altered in human hepatocellular carcinomas. The most frequently mutated genes of each pathway are also shown. The vertical arrowed lines connecting four pathways indicate that these pathways are related to each other and they should not be considered as independent and separate pathways of hepatocellular carcinogenesis.

β-CATENIN, *APC*, AND E-CADHERIN GENES

The *APC* gene was initially identified in the familial adenomatous polyposis coli syndrome. Germline and somatic mutations of *APC* have been detected in colorectal cancers.[26] Some of these cancers display mutations in the β-catenin gene instead of the *APC* gene.[27] *APC* and β-catenin proteins have physical and functional interactions.[28] β-Catenin also forms complexes with E-cadherin.[29] *APC* and E-cadherin may be involved in intercellular interactions.[28,29] In contrast, β-catenin appears to play a role in transcriptional regulation in addition to its participation in cell-to-cell interactions.[28] Somatic mutations of β-catenin were observed in 19–26% of HCCs.[30,31] These mutations that occur at the N-terminal region of β-catenin lead to an accumulation of aberrant β-catenin proteins that stimulate the activity of a transcription factor.[28,30,31] Somatic *APC* mutations may be rare in HCC, but they appear to be quite frequent in hepatoblastomas.[11,32–37] Finally, the E-cadherin gene was shown to display frequent LOH and *de novo* methylation in HCC (Table 1). Thus, it is possible that E-cadherin function is lost in some HCCs. Taken together, these observations indicate that the "β-catenin/*APC* pathway" is altered in more than 30% of HCCs.

OTHER GENETIC ALTERATIONS

As shown in Table 1, *ras* and *myc* oncogenes are not frequently mutated in human HCC. Loss of genomic imprinting and bi-allelic expression of the IGF2 gene was shown in hepatoblastomas and in some HCCs.[38–43] Among other known genes, *BRCA2*, *p21*, and *p15INK4B* appear to be involved only rarely in these tumors. *MLH1* and *MSH2*, two genes involved in DNA mismatch repair, have not been studied for possible mutations in HCC (Table 1).

CONCLUDING REMARKS

Recent studies clearly indicate that many genes undergo somatic aberrations (point mutations, amplifications, loss of imprinting, *de novo* methylation, etc.) in HCC. The number of aberrant genes is high, but the frequency of individual gene mutations is low. However, these mutations are not random. They tend to cluster at genes involved in important growth regulatory pathways. Even though the picture is still imperfect, our present knowledge of the molecular genetics of HCC leads us to four main pathways that are altered in HCC: the *p53* pathway involved in DNA damage response, the *RB1* pathway involved in cell cycle control, the TGF-β pathway involved in growth inhibition and apoptosis, and the β-catenin/*APC* pathway involved in morphogenesis and signal transduction. As illustrated in Figure 1, these pathways should not be considered as independent pathways. They are most probably related to each other and may even represent individually a distinct step of hepatocellular carcinogenesis. Unfortunately, our knowledge of the order of events for the initiation and stepwise progression of HCC is still incomplete.

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ABBREVIATIONS

HCC	hepatocellular carcinoma
HBV	hepatitis B virus
HCV	hepatitis C virus
M6P/IGF2R	mannose-6-phosphate/insulin-like growth factor II receptor
APC	adenomatosis polyposis coli gene
p16INK4A	gene coding for a 16-kDa inhibitor of cyclin-dependent kinase 4 enzyme
p15INK4B	gene coding for a 15-kDa inhibitor of cyclin-dependent kinase 4 enzyme
BRCA2	breast cancer susceptibility gene 2
HBx	X protein of hepatitis B virus
LOH	loss of heterozygosity
RB1	retinoblastoma gene
pRb	protein encoded by the retinblastoma gene
IGF2	insulin-like growth factor II
p19ARF	protein encoded by an alternatively spliced form of transcript from *p16INK4A* gene
MDM2	human homolog of mouse double mutant gene 2
p21	21-kDa cyclin-dependent kinase inhibitor protein also called CIP1
CDK4	cyclin-dependent kinase 4
E2F	a group of transcription factors regulated by the retinoblastoma family of pocket proteins
TGF-β	transforming growth factor β
MLH1	gene encoding a protein involved in DNA mismatch repair
MSH2	gene encoding for another protein involved in DNA mismatch repair

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